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CHLORIDE AND OSMOTIC BALANCE IN THE EURYHALINE SIPUNCULID *PHASCOLOSOMA ARCUATUM* FROM A MALAYSIAN MANGROVE SWAMP

JONATHAN P. GREEN AND DAPHNE FAUTIN DUNN[1]

Laboratory of Comparative Physiology, Department of Zoology, University of Malaya, Kuala Lumpur, Malaysia

Studies on the ability of members of the exclusively marine phylum Sipuncula to regulate their ion and water content have been admirably reviewed by Oglesby (1968, 1969). Although body volume may be controlled under conditions of low stress, virtually all studies point to the absence of osmoregulatory ability among sipunculids. The only possible exception is the species Oglesby (1968, 1969) calls *Phascolosoma lurco*. The high mangrove swamp habitat of this species, subject to extremely variable salinities (an atypical situation for a sipunculid), suggests that it might be able to regulate ions and/or volume, and Gross (1954), analyzing data from a study on this worm by Harms and Dragendorff (1933) (who referred to the species as *Physcosoma lurco*), concluded that this was indeed the case. However, the evidence is open to other interpretations, and Oglesby (1969, page 233) stated, "Detailed investigation of this interesting worm would be rewarding."

Phascolosoma arcuatum (Gray, 1828), as it is now known [for recent discussions of this species' taxonomy, see Rice and Stephen (1970) and Stephen and Edmonds (1972)], occurs from India to Queensland, Australia, presumably always in mud, in or near a mangrove swamp. Harms and Dragendorff (1933) repeatedly emphasized that the worms they studied in Ambon and Sumatra, Indonesia, were found only at the highest levels of the mangrove swamp, in mud covered by sea water only at extreme high tides. We collected specimens for study from areas subject to daily tidal inundation, as well as from high sites. In a mangrove swamp 25 miles south of our collecting site, Sasekumar (1974) found *P. arcuatum* at levels tidally wetted from 26 to 365 days a year, although the species was most abundant ($39/m^2$) in an area wetted 210 days per year (approximately mean high water spring). S. J. Edmonds (University of Adelaide, personal communication), who confirmed the identity of our worms, stated that in Queensland they live near mid-high water level.

We present here data from baseline measurements on the osmotic and ionic content of the coelomic fluid of freshly collected worms; from experiments on the effects of the type of media on the osmotic and ionic content of the worms' coelomic fluid; and from an experiment in which worms were subjected to a variety of salinities for up to 64 hours, designed to study short-term responses to sudden declines in environmental salinity, such as might occur during a tropical rainstorm.

[1] Current address: Department of Invertebrate Zoology, California Academy of Sciences, Golden Gate Park, San Francisco, California 94118.

Materials and Methods

Specimens of *Phascolosoma arcuatum* were collected from the vicinity of Kuala Selangor, about 50 miles northwest of Kuala Lumpur, Malaysia. Subjects for experiments were all taken from the same area of mangrove swamp at the mouth of the Selangor River, where it was not necessary to dig deeper than 20–30 cm to find abundant animals. On one occasion coelomic fluid was drawn from the worms at the collection site and returned to the laboratory in individual tubes on ice. Thereafter intact worms were taken to the laboratory in a bucket of mud, no more than two hours elapsing between collection and arrival at the laboratory. Sampling was done, or experiments were begun, no more than 18 hours after collection, unless otherwise stated.

Mud was rinsed from the worms with tap water, the animals were blotted dry and weighed on a Mettler top loading balance (P160N) to the nearest 0.1 g. Worms less than one gram in weight contained insufficient coelomic fluid for our purposes; most specimens weighed between one and four grams, but some were as heavy as nine grams. Coelomic fluid was drawn into a syringe through a 25 gauge needle, the needle removed, and the contents dispensed into a plastic centrifuge tube. Samples were centrifuged for five minutes in a refrigerated Sorval SS-1 centrifuge at 3100 *g*. The plasma was decanted and frozen in small plastic vials at −12° C until analyzed. In a number of samples some of the fresh fluid was drawn into a capillary tube and centrifuged at room temperature for five minutes in a hematocrit centrifuge. The length of the constituents in the tube was measured under a dissecting microscope and the percentage of formed elements calculated.

Chloride ion content, reported as milliequivalents per liter (meq), was determined by titration with 0.1 N silver nitrate (British Drug House Concentrated Volumetric Solution), using a potassium chromate indicator. Usually three but as many as five determinations were made on each sample to obtain a mean value. Osmotic concentration, reported as milliosmoles per liter (mosmol), was determined from measurements made on a Hewlett-Packard 302B Vapor Pressure Osmometer (VPO). A mean value was determined from a minimum of three but occasionally from four or five readings on a single sample. An effort was made to perform both chloride ion and osmotic concentration determinations on each sample. Conversion from titration or VPO values to chloride ion or osmotic concentration was by reference to a least squares linear regression line calculated from a series of sodium chloride solutions of known activity.

For the 64 hour experiment, about 160 freshly collected, rinsed animals were weighed and fluid was drawn from ten worms of various sizes. The others were put into individual plastic cups containing 100 ml 100% standard sea water [standard (artificial) sea water: NaCl 24.72 g/l; KCl 0.67 g/l; $CaCl_2 \cdot 2H_2O$ 1.36 g/l; $MgCl_2 \cdot 6H_2O$ 4.66 g/l; $MgSO_4 \cdot 7H_2O$ 6.29 g/l; $NaHCO_3$ 0.180 g/l (Cavanaugh, 1956)]. After 24 hours of equilibration they were reweighed, coelomic fluid was drawn from five specimens of various sizes, and the rest, still in individual cups, were divided into 35 groups of four worms each. An effort was made to have a distribution of worms of similar weights in each group. Seven groups of worms were covered with 100 ml of water of each of five salinities (100, 75, 67, 50, and 40 per cent of standard sea water). Coelomic fluid was drawn from one group in

each salinity 1, 2, 4, 8, 16, 32, and 64 hours later. All remaining worms were weighed at each sampling time, as well as at 24 and 48 hours when their water was changed. The cups were covered, more to prevent escape than evaporative loss, and the experiment was done in an air-conditioned laboratory ranging in temperature from 25.5 to 27.5° C.

All values are reported as a mean ± one standard deviation, and statistical significance is determined by Student's *t*-test, except where otherwise noted. In the few instances that the standard deviation exceeded 10% of the mean, the divergent value was eliminated from consideration and the statistics were recalculated. In some cases we found that the worm showing such deviation from others subjected to identical conditions had appeared abnormal.

Results

Baseline data

The coelomic fluid of *Phascolosoma arcuatum* does not clot when drawn. Although usually pink in color, the fresh fluid of several animals bled in the field was yellow. The percentage of formed elements in the fluid of 35 worms collected on four different occasions was 4.3 ± 2.3%, with a range of 2.2 to 12.8%, and after centrifugation formed two light colored layers bracketing a thicker layer of red colored cells. Data from the occasional animals in which greenish eggs were present in the coelomic fluid are not included.

Chloride ion and osmotic content of coelomic fluid of six batches of sipunculids are related to that of their media in Table I. In the first group listed, worms were collected on the bank of a drainage ditch across the main road and several hundred meters from the Selangor River, but the water came from the ditch itself. In all other instances, the animals were from typical mangrove swamp habitats and seep water from the same microhabitat was collected for comparison. In the fourth batch listed, coelomic fluid was drawn 18, 24, and 48 hours after collection, but since readings on the three were stastically equal, the results from all samples were combined.

Table I

Chloride and osmotic content of coelomic fluid from freshly collected sipunculids and water from their immediate environment. Each line represents a single collection.

mosmol		Number of worms	CF/M	Significance of difference $P \leq$	meq Cl^-		Number of worms	CF/M	Significance of difference $P \leq$
Medium (M)	Coelomic fluid (CF)				Medium (M)	Coelomic fluid (CF)			
*342.7	632.5 ± 49.2	10	1.85	0.01	*138.2	240.2 ± 14.5	10	1.74	0.01
378.5	625.5 ± 50.5	8	1.65	0.01	192.3	335.2 ± 27.1	9	1.74	0.01
559.7	731.4 ± 31.8	7	1.31	0.01	253.4	349.4 ± 6.9	5	1.38	0.01
675.9	749.7 ± 65.3	15	1.11	n.s.	283.3	269.4 ± 24.0	18	0.95	n.s.
830.2	759.3 ± 38.8	17	0.91	n.s.	359.0	369.6 ± 14.9	21	1.00	n.s.
738.9	804.7 ± 35.8	5	1.09	n.s.	422.4	395.9 ± 39.3	6	0.94	n.s.

* Not seep water; see text.

TABLE II

Chloride and osmotic content of coelomic fluid of worms and their medium after being kept in either mangrove swamp water or mud, or in artificial sea water. Variances were compared by Bartlett's test for homogeneity of variances, the χ^2 values of which are indicated between the two values compared.

		mosmol					meq Cl^-				
		Medium (M)	Coelomic fluid (CF)	Number of worms	CF/M	Significance of difference $P \leq$	Medium (M)	Coelomic fluid (CF)	Number of worms	CF/M	Significance of difference $P \leq$
24 hr	water mud	718.6	415.1 ± 21.7	3	0.58	0.01	215.2	198.5 ± 10.6	8	0.92	n.s.
		$\chi^2 = 1.69$ (ns)					$\chi^2 = 1.26$ (ns)				
		793.2	765.1 ± 46.1	6	0.96	n.s.	345.4	337.3 ± 14.7	6	0.98	n.s.
48 hr	water mud	731.3	701.9 ± 6.0	4	0.96	0.05	356.9	330.2 ± 8.3	5	0.93	0.05
		$\chi^2 = 10.37$ ($P < 0.01$)					$\chi^2 = 0.08$ (n.s.)				
		563.6	546.6 ± 37.0	4	0.97	n.s.	245.9	228.6 ± 9.1	6	0.93	n.s.
24 hr	artificial sw	897.5	885.7 ± 36.4	5	0.99	n.s.	461.5	476.1 ± 10.4	4	1.03	n.s.
48 hr		1016.3	932.2 ± 54.7	5	0.92	n.s.	484.0	448.6 ± 38.4	4	0.93	n.s.
		1075.1	946.8 ± 14.0	8	0.83	0.01	428.5	457.4 ± 8.3	7	1.07	0.02

In another two cases, worms but not water were collected. Chloride ion content of these was 411.1 ± 31.5 meq (nine worms) and 493.5 ± 38.9 meq (20 worms). VPO measurements were 831.7 ± 51.1 mosmol (eight worms) and 990.4 ± 68.9 mosmol (18 worms), respectively.

Mud versus water medium

Experiments were done on two occasions to determine whether any osmoregulatory ability that might exist would be affected by keeping worms in water rather than in mud. Sipunculids, mud, and seep water were collected at the same time and place. In the laboratory the worms were rinsed and randomly placed for 24 hours in one case or 48 hours in the other, either in mangrove swamp water or in mud saturated with the same water. Among the specimens collected for the 48 hour experiment, some were bled immediately upon return to the laboratory. The upper part of Table II summarizes the findings of these experiments. Results of three other experiments, in which worms were kept in 100% standard (artificial) sea water for 24 or 48 hours, are summarized in the lower part of Table II.

In addition to the application of Bartlett's test for homogeneity of variances (Sokal and Rohlf, 1969) between coelomic fluid of experimental subjects as indicated in Table II, this test was used to compare variances between these fluids and those of fresh worms. The variance of the chloride values of neither the mud nor the water group of 48 hour worms differs significantly from that of fresh sipunculids ($\chi^2 = 0.27$ and 0.73, respectively), but variance in the VPO values of those kept in mud is significantly less ($P < 0.01$) than that of fresh specimens ($\chi^2 = 12.57$). Variance in coelomic fluid VPO values of fresh worms does not differ significantly

from that of worms kept in water 48 hours (next to bottom line, Table II; $\chi^2 = 0.00$ and 1.40, respectively). In the 24 hour group, variance in VPO measurements between fresh and 24 hour coelomic fluid does not differ significantly ($\chi^2 = 1.26$), but the variance of the chloride ion values is significantly greater (at the 0.02 level; $\chi^2 = 5.79$) in coelomic fluid from fresh worms.

Salinity/time experiment

During the 24 hour equilibration period prior to the experiment, during which the worms voided most of their gut contents, 132 worms lost 22.3 ± 5.4% of their body weight, with losses ranging from 8.0 to 37.0%. Those weighing less than two grams after the 24 hours lost 23.6 ± 5.4% (N = 73), those in the range 2.0–4.4 g lost 21.5 ± 5.0% (N = 50), and those 4.5 g and more lost 17.5 ± 3.8% (N = 11) of their original body weight, the differences being significant.

Figures 1–3 and Table III present the results of this 64 hour experiment. To determine time to equilibrium, values from successive times were compared and in those cases where there was no difference at the 0.05 level of significance, alternate times were compared at the 0.02 level of significance. Worms were considered to have reached equilibrium when no significant differences were detected according to these criteria. For chloride ion and osmotic concentration readings, the mean value of all worms having attained equilibrium (*i.e.*, all worms at equilibrium time and all later times) was then compared with the value of the medium. The only exception to this was in the chloride ion concentration of worms kept in

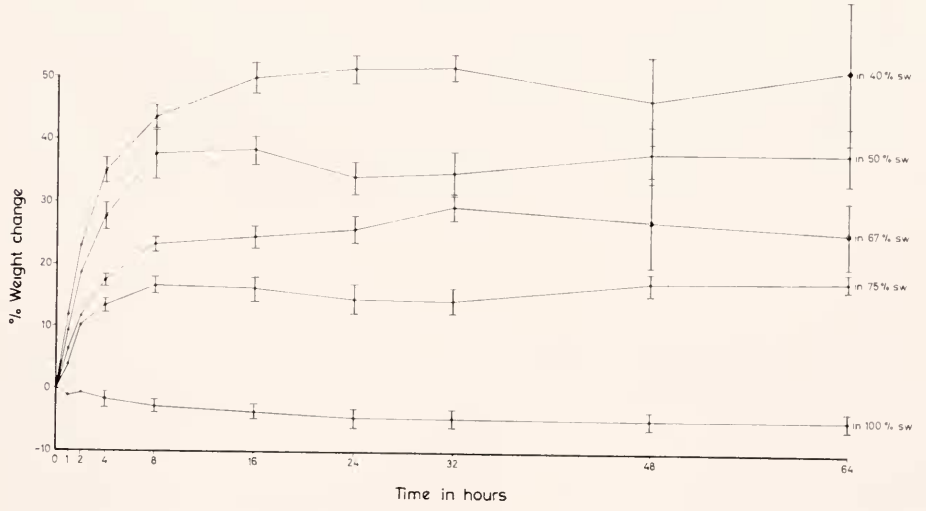

FIGURE 1. Change in weight of specimens of *Phascolosoma arcuatum* maintained in the laboratory for up to 64 hours in water of different salinities. One hour values are based on 22 worms in 100% sea water, 26 worms in 75% sw, 27 worms in 50% sw, and 28 worms in 40% sw. Numbers decline by three or four at each sampling time, and circled points represent a value obtained from only two worms. Standard error bars are omitted below four hours but are of a similar magnitude to the later ones.

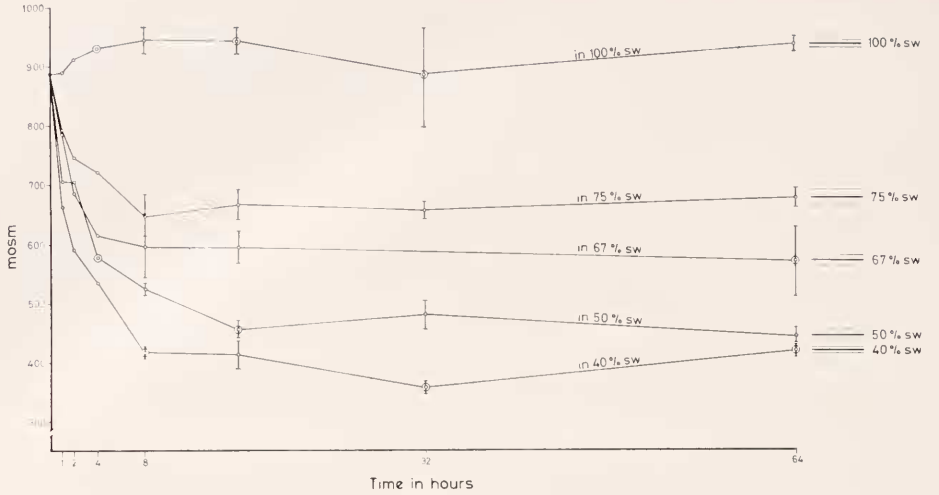

FIGURE 2. Changes in osmotic content of *Phascolosoma arcuatum* coelomic fluid with time, in water of different salinities. Circled points are means of measurements on two worms; other points are means of measurements on three or four worms. Standard deviation bars are omitted below eight hours but are of a similar magnitude to the later ones.

100% sea water. Values did not change significantly between successive times but the readings at two and eight hours differed at the 0.01 level of significance. Nonetheless a mean equilibrium value was calculated from all the readings. Similarly, the mean 100% osmotic concentration value was calculated from all readings (1–64 hours) since there was no significant change (neither between successive nor alternate times) through the course of the experiment. The osmotic concentration of worms kept in 40% sea water never attained a stable equilibrium, but all others did. Although a few of the experimental subjects escaped or died during the course of the experiment, the losses were not correlated with salinity.

DISCUSSION

The yellow color of the coelomic fluid in a few of the animals bled in the field was due to the deoxygenated state of the hemerythrin. The pink fluid of all those bled in the laboratory reflected the oxygenated condition of the pigment, showing that the time (in some cases only a few hours) between removal from the mud and bleeding was sufficient for oxygenation to occur. After centrifugation, the fluid was usually clear and colorless, although in a few cases it was cloudy and yellowish, but there was no correlation between its appearance and its osmotic character. Nor was there a correlation between the formed element content of a worm's coelomic fluid and its osmotic character.

Individual baseline measurements ranged from 189 meq Cl^- and 396 mosmol to 469 meq Cl^- and 963 mosmol, and up to 571 meq Cl^- and 1135 mosmol in animals along with which no seep water was collected for comparison. The proportion of the total osmotic concentration accounted for by chloride is quite variable.

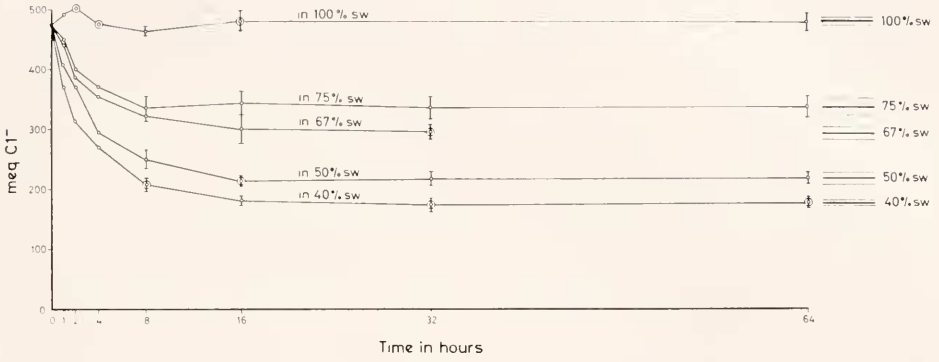

FIGURE 3. Changes in chloride ion content of *Phascolosoma arcuatum* coelomic fluid with time, in water of different salinities. Circled points are means of measurements on two worms; other points are means of measurements on three or four worms. Standard deviation bars are omitted below eight hours but are of a similar magnitude to the later ones.

Harms and Dragendorff's (1933) baseline values ranged from 470 to 1040 mM NaCl, although it was not always clear whether the value was obtained from the coelomic fluid of a single worm or from a pooled sample. Although such a range is apparently not found in natural populations of other species of sipunculids so far studied, Oglesby (1968) reported a range of approximately 100 to 600 mM NaCl for groups of the stenohaline sipunculid *Themiste dyscritum* after laboratory adaptation to various salinities.

The osmotic and chloride ion content of collected seep water varied from about 42 to 92% of standard sea water (excluding water values in the first line of Table 1). *Phascolosoma arcuatum* is hyperosmotic and hyperionic in waters as concentrated as 253 meq Cl^- (55% standard sea water), and 560 mosmol (62% standard sea water). In more concentrated media, there is no significant difference between chloride or osmotic content of coelomic fluid and that of the medium, although data from two of the three groups indicate that the worms may be hyperosmotic even at higher salinities. Coelomic fluid of most of the worms studied by Harms and Dragendorff (1953) was hyperosomotic to the medium. Curiously,

TABLE III

Time to equilibrium after 24 hours pretreatment in 100% sea water, and ratio of coelomic fluid to medium osmotic and chloride ion concentration at equilibrium.

Percentage of sea water	Weight	Osmotic concentration		Chloride ion concentration	
	Hours	Hours	CF/M	Hours	CF/M
100%	—	—	1.02	—	1.04
75%	8	8	0.98	8	0.97
67%	8	2	1.00	8	1.01
50%	8	16	0.99	8	1.00
40%	16	—	—	16	1.02

it appears that conforming worms can have a lower chloride content than those in which the Cl^- level is significantly greater than that of the medium (*e.g.,* compare the fourth batch, Table I, with the preceding two). Further studies are necessary to clarify these points.

In the time/salinity experiment, on the other hand, worms at all experimental salinities reached equilibrium well within 64 hours, at which levels osmotic concentration (except for the 40% group) and chloride ion content did not differ significantly from those of the media. In fact, although our 100% sea water was more concentrated than any of the waters collected *in situ,* 24 hours in it was sufficient for the worms to conform to it, as evidenced by the lack of significant change in Cl^- and VPO values in the 100% groups of worms during the succeeding 64 hours of the experiment.

The discrepancy between isosmosity of coelomic fluid of nearly all groups of experimental worms and hyperosmosity of coelomic fluid of at least some groups of freshly collected animals may be in part a reflection of methodological problems. Seep water might not accurately reflect the immediate osmotic environment of the worms, a possibility also discussed by Harms and Dragendoff (1933). They repeatedly emphasized that *P. arcuatum* lives in air-filled tubes so that the worms are not actually in contact with the ground water, adding that the worms avoid water and cannot even be kept in it because they are adapted to aerial respiration. The deoxygenated hemerythrin that occurred in some of our worms bled in the field suggests that their tubes were probably water-filled. Possibly the coelomic fluid hyperosmosity was due to desiccation, as suggested by Oglesby (1969, page 233), since all our specimens were collected at low tide, but if this were the explanation, the phenomenon probably would be evident at all salinities. It is also possible that at low tide, water from the near-by Selangor River percolated through the mud, lowering the salinity of the seep water. Thus worms collected at this time may not have reached equilibrium.

Since it is easier to define salinity for experimental purposes in an aqueous medium than in mud, and since we could be certain that in water the animals would be in contact with the medium we had defined, we preferred to use a water medium, providing it did not interfere with the worms' normal physiological processes. In the mud versus water medium experiments, only one of five groups of worms kept in water differed in chloride and osmotic content from their medium, whereas comparable values for coelomic fluid of the two groups kept in mud were both lower than water derived from the mud (although the difference was not significant for Cl^- of the 24 hour group). If the sipunculids were "drowning" in water, their physiological integrity might be expected to deteriorate, but there was no evidence of this. In only one instance was a significant difference found between the variances of coelomic fluid measurements of those kept in water and of those kept in mud, but comparison with fresh coelomic fluid of worms collected at the same time showed no difference in variances of osmotic content with that of worms kept in water for 48 hours. Thus the variability of the coelomic fluid of those animals kept in water was not greater than usual, but that of those kept in mud was less than usual. The only other instance of significantly different variances was that Cl^- of fresh worms was greater than that of worms kept in artificial sea water for 24 hours, but variances in osmotic concentration of the same worms were statistically equal.

Thus it appears that at least for the length of time involved in these studies, immersion in water does not affect the osmotic physiology of *Phascolosoma arcuatum*. Indeed, as it turned out, nearly all worms in the 64 hour experiment showed normal muscle tone and activity even in 40% sea water and for up to 64 hours.

Results of the 64 hour experiment clearly indicate that *P. arcuatum* can be an osmo- and chloride ion-conformer, but it is also apparent from Table II that a mud medium may permit the worms to differ from their environment, assuming that water obtained by centrifuging this mud is an accurate reflection of that environment. For example, seep water of the third group in Table I is of nearly the same salinity as that of water in the 48 hour mud/water experiment (Table II) but coelomic fluid of the former worms is significantly higher in both parameters than the medium whereas in the latter they are statistically equal. Tubes built by worms in mud might isolate the animals from the seep water. *P. arcuatum* is a deposit feeder and freshly collected worms always had guts filled with mud. Our data suggest that the presence of substrate within and without the animal may modify the organism's response to osmotic stress. A mud-filled gut might serve as a buffer against changes in the external environment, perhaps being especially important during short-term changes in environmental salinity, such as during rainstorms at low tide or during a period of unusually high tides. An assessment of the relative roles of body wall and gut wall in water and ion transport is clearly needed.

The fact that time to ionic equilibrium in the 64 hour experiment was roughly the same as the time to weight equilibrium suggests that the movement of water was responsible for the adjustment. In contrast with the situation in *Physcosoma japonicum* (Koller, 1939) where worms transferred to lower salinities return to their original weight within 30 hours after an initial 10 hour period of weight gain, in *P. arcuatum* there was no return to some "baseline" level. Our data conform to Gross' (1954) generalization that time to osmotic equilibrium in sipunculids is directly related to the extent of the osmotic stress, and suggest that large worms take longer to reach equilibrium as also noted by Gross, although numbers of animals in each time/salinity group were too small to analyze statistically for differences that might be due to their weight. Our finding that during the 24 hour equilibration period, larger worms lost proportionately less weight, suggests that there are probably differences in osmotic behavior in worms of various sizes. (This initial weight loss was due in part to voiding of feces. However, the 100% sea water in which the worms were acclimated was more concentrated than that from which they had been collected and since, as it turned out, the worms were osmo-conforming, presumably part of the weight loss was due to water loss.) Further evidence for this is that, of the ten values that were discarded in the 64 hour experiment because they made the s.d. greater than 10% of the mean, seven were from the largest worm of a group of four, and all were values much lower than the others of the same group.

As mentioned in the introduction, *Phascolosoma arcuatum* occupies a much wider intertidal range in Malaysian mangrove swamps than is reported by Harms and Dragendorff (1933) for the species in Indonesia. The fact that they used individuals collected so high in the swamp may account for some differences between their observations and ours. In addition, their sample sizes were very small, limiting the usefulness of the study. Results of our research lead us to conclude that

Phascolosoma arcuatum, like all other sipunculids studied in this way, is physiologically an osmoconformer, and thus, in water, this species is really no different from *Themiste dyscritum,* which is also tolerant of and can conform to salinities ranging from about 40 to 100% (Hogue and Oglesby, 1972), although the former is naturally exposed to such a variable environment and the latter is not. However, under natural conditions, methods of behaviorally manipulating its environment may be a factor in allowing *P. arcuatum* to be hypersomotic to its environment, at least at low salinities, and this distinctive feature probably contributes to this species' ability to live in its atypical habitat.

We are grateful to Prof. A. J. Berry for introducing us to *Phascolosoma arcuatum,* to Dr. S. J. Edmonds for identifying the worms, to Mr. M. Arunasalam for digging them, and to Mr. Amir bin Salleh for drawing the graphs. Drs. A. Pritchard, Y. C. Siew, R. I. Smith, and G. C. Whittow gave helpful suggestions. This work was supported by a research grant from the University of Malaya.

Summary

1. The euryhaline sipunculid *Phascolosoma arcuatum* (Gray, 1828) occurs from lowest to highest levels of mangrove swamps along the west coast of Peninsular Malaysia.

2. The chloride ion content of coelomic fluid of freshly collected *P. arcuatum* varied from 189 to 571 meq Cl^-, and the total osmotic content varied from 396 to 1135 mosmol. The lower concentrations were hyperionic and hyperosmotic to the surrounding water, but the higher concentrations of coelomic fluid were isoionic and isosmotic.

3. Animals kept for up to 64 hours in the laboratory in artificial sea water ranging from 40 to 100% standard sea water were uniformly isoionic and isosmotic at equilibrium. Weight stabilized at about the same time as did ionic and osmotic content of the coelomic fluid, without evidence of being regulated.

4. Under natural conditions, mud in the worms' guts and around them may act as a buffer, thereby allowing the animals to maintain an ionic and osmotic state differing from that of their environment.

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